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THE ONTARIO WATER RESOURCES COMMISSION

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THE ENUMERATION OF BACTERIOPHAGES IN WATER AND SEWAGE USING THE MOST PROBABLE NUMBER METHOD

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INTRODUCTION

Bacteriophages ('phages) are viruses which infect and usually destroy, living bacteria. Certain types of 'phages are pathogenic on bacteria which occur in human feces. These 'phages may be useful as indicators of human enterovirus survival in water and sewage. A method of enumeration of very low numbers of such 'phages was described by Kott (1). It was relatively unaffected by the turbidity or degree of contamination of the sample; furthermore, titres were obtained which closely corresponded to the actual number of 'phage particles present, as determined by the conventional soft agar technique (2). Preliminary experiments employing the method had also indicated that standard 'phage assay broth (2) could be substituted as a growth medium.

The only apparent drawback to the method was that the plaques formed during the detection of 'phage, were sometimes obscured by an overgrowth of contaminating organisms, especially in sewage or polluted samples. Some workers recommended heating the growth tubes at 56°C for 30 minutes prior to plating out to detect 'phage (3). However, experiments using this technique had revealed that heating also inactivated the 'phage to some degree. In this phase of the study, media selective for coliform organisms were investigated for their ability to minimize

this overgrowth of contaminating organisms and yet not affect the titre of 'phage obtained. Certain selected water samples were also studied to determine the usefulness of the method in monitoring water samples for virus pollution.

MATERIALS AND METHODS

Cultures

The E coli B (Dept. of Microbiology, School of Hygiene, University of Toronto) was maintained in nutrient agar slopes at room temperature, transferred monthly.

The 'phage had been isolated from sewage and prepared in pure culture according to the method of Adams (2) (see report 2010).

Media

A) 'Phage assay broth (PAB) this was the control medium to which all others were compared, being equivalent in performance to the medium described by Kott without the addition of $CaCl_2$. It consisted of 8gm nutrient broth (Difco) and 5gm NaCl dissolved in 1 litre distilled water.

B) Lauryl tryptose broth (Difco) made up according to directions.

C) MacConkey broth (Difco) made up according to directions.

D) Lactose broth (Difco) made up according to directions.

'Phage was detected on solid 'phage assay agar (PAA) made up of 8gm nutrient broth, 5gm NaCl, 15gm agar per litre of distilled water.

Experimental

Each of the four media were set up in the following series:

5 tubes of 10 ml double strength medium plus 10 ml sample

5 tubes of 10 ml single strength medium plus 1 ml sample

5 tubes of 10 ml single strength medium plus 0.1 ml sample.

Each series was inoculated with the same sample, which contained approximately 110 'phage particles per 100 ml as determined by the agar layer technique.

Each tube was then plated out to determine the presence or absence of 'phage, on to PAA in the normal way. The combination of 'phage positive tubes in each series was then applied to the probability tables, to give the number of 'phage particles per 100 ml of sample.

The results for the three selective media compared to the PAA control are shown in Table I. Of the three media, lauryl tryptose broth appears to yield titres which are greater even than those obtained with the PAA. However, they are still of the same order of magnitude as the actual titre, especially when the lower limits are considered.

Since the method was to be applied to the enumeration and detection of small numbers of 'phage, lauryl tryptose broth would appear to be the medium of choice, since it seems to have the effect of enhancing the titre. As far as decreasing the effect of contaminating organisms in the samples is concerned, none of the media tried offered any great advantage over PAB.

Calcium chloride ($CaCl_2$) is known to have a stimulatory effect on some 'phages, and Kott included it in his original medium. Thus, $CaCl_2$ was added to the optimum medium ie. lauryl tryptose broth, to determine whether any effect on the titre could be obtained. The lauryl tryptose broth was made up as directed, and sterile $CaCl_2$ solution, to give a concentration of 0.15 gm/litre was added immediately before use.

The effect of the addition is shown in Table II. The titre is consistently greater in the presence of $CaCl_2$, than in its absence, although the limits of the titres fall in essentially the same range.

During the course of this study, selected samples from municipal water works, comprising both treated and untreated waters, were examined for the presence of 'phage. At no time was 'phage detected using this method, even though low levels of coliforms were present in some of the samples. Samples of water known to be polluted to some degree,

including lagoon and activated sludge effluents and water from receiving streams, were also examined; such samples were found to contain 'phage, but coliforms were also invariably present. 'Phage counts, in agreement with findings on sewage-polluted shell fish (4), were lower than, but parallel to coliform counts.

DISCUSSION

Lauryl tryptose broth (Difco) with added $CaCl_2$ yields 'phage titres superior to those obtained in the medium originally described for this method. It has the advantage that it can be made from a standard, reproduceable dehydrated medium, the only addition being the calcium chloride solution. The increased 'phage titres may partly be due to the property of the selective medium, with respect to an enhancement of the growth of the host organism (E coli B), over other contaminating organisms. The $CaCl_2$ may be a stimulatory factor when dealing with 'phages that require the presence of Ca++ions for reproduction.

The method would appear to offer no great advantage over the standard coliform count in the monitoring of water supplies for enteroviruses, since at no time was 'phage detected in the absence of coliforms. Also, the resistance of 'phage to external conditions is apparently less than, although similar to, these viruses (5).

However, Serratia marcescens 'phage is actually more resistant than poliovirus (6). The MPN method described here could be readily adapted to enumerate this 'phage, which could be used as an indicator of enterovirus, in studies such as those to determine the efficacy of a given treatment process in removing such viruses.

This technique does however, allow enumeration of a specific organism of fecal origin, since 'phage would not be present without the prior occurrence of its host E coli B. The method could therefore prove useful, as has previously been suggested (7), in monitoring E coli B 'phage as an indicator of pollution of fecal origin.

The method has found application in specialized circumstances where low levels of 'phage must be detected, and the standard agar layer procedure is inapplicable. The latter is accurate at levels of 10 - 30 'phage particles per ml, whereas the theoretical lower limit of the MPN method is 2 'phage particles per 100 ml.

SUMMARY

A substitute medium for use in the MPN method for the enumeration of 'phage is described. The significance of the results of the examination of several types of water samples for the presence of 'phage is discussed.

TABLE I

Titre of 'phage sample suspension obtained in different test media using the MPN method.

Medium	Titre/100 ml						Actual titre/ 100 ml+
	Test 1		Test 2		Test 3		
	MPN	Limits	MPN	Limits	MPN	Limits	
'Phage assay broth control	130	35-300	79	25-190	190	31-150	110
MacConkey broth	23	7-70	0*		0**		110
Lactose broth	5	<0.5-13	13	3-31	4	<0.5-11	110
Lauryl tryptose broth	348	120-1000	542	180-1400	542	180-1400	110

* no 'phage detected

** number of 'phage could not be determined from the combination of positive tubes.

\+ determined by agar layer method

TABLE II

The titre of 'phage suspensions as determined by the MPN method in the presence and absence of Calcium chloride, using Lauryl tryptose broth.

Test	Titre/100 ml				Actual titre/100 ml
	+ $CaCl_2$		- $CaCl_2$		
	MPN	Limits	MPN	Limits	
1	79	25-190	70	23-170	55
2	348	120-1000	49	17-120	55
3	11	2-25	8	1-19	11.0
4	49	17-130	11	2-25	11.0
5	278	90-850	49	17-130	55

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